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Selection of DNA Aptamers for Breast Cancer

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Abstract—A method of selection of DNA aptamers to breast tumor tissue based on the use of postoperative material has been developed. Breast cancer tissues were used as the positive target; the negative targets included benign tumor tissue, adjacent healthy tissues, breast tissues from mastopathy patients, and also tissues of other types of malignant tumors. During selection a pool of DNA aptamers demonstrating selective binding to breast cancer cells and tissues and insignificant binding to breast benign tissues has been obtained. These DNA aptamers can be used for identification of protein markers, breast cancer diagnostics, and targeted delivery of anticancer drugs.

Kewords: SELEX, DNA aptamers, oligonucleotides, breast cancer

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INTRODUCTION

Breast cancer is the leading cause in the structure of malignant tumor morbidity. The mortality rate reaching 40% [1, 2] is obviously associated with the lack of available methods for early diagnosis, as metastases that occur at the later stages of this disease make the major contribution to mortality [3]. Breast cancer diagnostics is mainly based on invasive procedures, as histological studies still remain the standard of diagnostics of this disease. However, mass screening of population requires simple and minimally invasive diagnostic methods, for example, based on detection of tumor markers in biological fluids. Various proteins are used for diagnostics of breast cancer. These include carcinoembryonic antigen CEA, the Ki-67 antigen (a cell cycle and tumor growth marker), epidermal growth factor receptor EGFR HER-2 [4, 5], mammaglobin MGBA, factors of epithelial-mesenchymal transition and metastasis (Snail, Twist, Zeb1) [6]. However, these markers do not have a sufficient level of sensitivity and specificity [4–6].

Search for tumor markers is a complex multi-stage process based on the comparison of proteomes of normal and malignant tissue, which does not necessarily give positive results [7]. This possibly explains lack of reliable molecular markers employed for diagnostics of breast cancer in clinical practice [4–6]. Another approach for the search for tumor markers is based on a technology that allows to use aptamers for identification of tumor-associated proteins [8].

Aptamers are short synthetic single-stranded RNA or DNA molecules, which can bind to functional groups of their biological targets with high affinity and selectivity due to their unique three-dimensional conformation; being functional analogs of protein antibodies they have a number of advantages [9]. Aptamers selection based on screening of a large number of sequences from oligonucleotide libraries in vitro or in vivo, results in select of highly specific aptamers to any target, from small inorganic ions to intact tissue [10–13]. Knowing nucleotide sequence, a selected pool of aptamers can be further amplified and chemically synthesized. Using aptamers that bind only to proteins of tumor tissues, it is possible to isolate and identify these proteins and use them for diagnostics of cancer [14].

In this study we have selected a pool of DNA aptamers that can selectively bind to breast cancer cells and tissues. The selection procedure included breast cancer tissues, benign breast tumor tissue, adjacent healthy tissues, breast tissues from mastopathy patients, and also tissues of other types of malignant tumors obtained during surgical operations.

MATERIALS AND METHODS

Selection of DNA Aptamers

DNA aptamers to breast cancer tissues were selected by using the tissue-SELEX technology [14] and a single-stranded Harvard library of DNA aptamers [15] with the 40-nucleotide variable region and

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primer sites from the 5' and 3' ends: CTCCTCTGACTGTAACCACG (N) (N1)(N)(N1) (N)(N1)(N) (N1)(N2)(N2) (N2)(N2)(N) (N1)(N)(N1) (N)(N1)(N) (N1)(N)(N1) (N2)(N2)(N2) (N)(N1)(N) (N1)(N)(N1) (N)(N1)(N) (N1)(N2)(N2) (N2)(N2)(N) GCATAGGTAGTCCAGAAGCC, where N is mixture of nucleotides at a ratio of A/C/G/T = 45 : 05 : 45 : 05, N1 is mixture of nucleotides at the ratio of A/C/G/T = 05 : 45 : 05 : 45, and N2 is a mixture of nucleotides at the ratio A/C/G/T = 25 : 25 : 25 : 25. Such improved structure of the library increases diversity of hairpin conformations compared with standard randomized libraries. During positive selection 13 samples of postoperative breast tumor tissues from different patients were used. The negative selection employed 10 samples of benign tumor tissue, one tissue sample with mastopathy, one sample of lung cancer tissue, and one glioblastoma tissue sample.

This study was carried out in strict accordance with the documents governing the ethical aspects of research using biological material of human origin (decision of the Ethics Committee at Voino-Yasenetski Krasnoyarsk State Medical University no. 37/2012 of 31.01.2012, decision of the Ethics Committee at KKRCCC no. 8/2011 of 16.03.2011).

Tissue samples removed during surgery were placed in Hank's medium. After 3 h tissue (0.5–1 cm^3) was washed three times with 5 mL of 10 mM phosphate buffer (pH 7.4) containing 3 mM Ca^{2+} and Mg^{2+} (final concentration), was suspended, washed three times, and diluted in 100 μL of 10 mM phosphate buffer (pH 7.4).

DNA aptamers were selected during 11 rounds of positive and negative selection as described earlier [14]. Selection was performed in PBS supplemented with divalent calcium and magnesium ions (final concentrations of 3 mM). The first round included only positive selection towards breast cancer tissues; briefly, 50 μL of the tissue suspension was incubated with yeast RNA (final concentration 1 mg/mL) in 100 μL of phosphate buffer (pH 7.4) containing Ca^{2+} and Mg^{2+} for 30 min at room temperature. After this incubation the FAM-labeled DNA library (final concentration of 1 μM) was added to the cell suspension (protected from light), and this mixture was then incubated at room temperature on a shaker for 30 min. The suspension was further centrifuged at 5000 *g* for 5 min; the supernatant was removed and the sediment was washed three times with the phosphate buffer containing Ca^{2+} and Mg^{2+} to remove unbound oligonucleotides. In order to increase the stringency of aptamer selection, the volume of the washing buffer was increased during each round by 50 μL, starting with 0.5 mL of the first and reaching 1 mL during the final round. Aptamers bound to the cells (and remained in the sediment after washing) were separated from their targets by denaturation in 10 mM Tris-EDTA buffer (pH 7.4; 75 μL) for 5 min at 95°C followed by cooling on ice for 5 min. Then the supernatant containing the candidate aptamer pool obtained after centrifugation for 15 min at 13000 *g*, was taken into a separate tube. The number of copies of sequences selected in the round were amplified by symmetric polymerase chain reaction (PCR), followed by asymmetric PCR for subsequent preparation of single-stranded DNA aptamers from the double stranded PCR product.

Symmetric PCR was performed using 5 μL of the aptamer pool in TE buffer and 45 μL of the reaction mixture. During the first three rounds the following amplification mixture was used: PCR buffer A (KAPA Biosystems, USA), 2.5 mM $MgCl_2$, 0.025 U/μL KAPA2G HotStart Robust polymerase (KAPA Biosystems), 220 μM dNTPs, 300 nM forward primer (5'-CTCCTCTGACTGTAACCACG-3') and 300 nM reverse primer (5'-GGCTTCTGGACTACCTATGC-3'). Starting from the fourth round, the mixture was modified to enhance reaction efficiency: standard PCR buffer A was replaced for PCR buffer B (KAPA Biosystems) for DNA amplification (from a complex multicomponent media) supplemented with Enhancer 1 A KAPA Biosystems solution used at the ratio buffer B : Enhancer 1 = 1 : 1.

In the asymmetric PCR used to obtain single-stranded DNA concentration of the forward primer was 20 times higher than that of the reverse primer. The reaction was performed using 5 μL of the symmetric PCR product and 45 μL of a mixture for asymmetric PCR: PCR buffer, 2.5 mM $MgCl_2$, 0.025 U/μL KAPA2G HotStart Robust polymerase, 220 μM dNTPs, 1 μM of FAM fluorescently-labeled forward primer (5'-/FAM/CTCCTCTGACTGTAACCACG-3') and 50 nM reverse primer (5'-GGCTTCTGGACTACCTATGC-3'). Symmetric and asymmetric PCR reaction was performed using the following program: preheating for 2 min at 95°C, followed by 15 cycles for 30 s at 95°C, 15 s at 56.3°C and 15 s at 72°C. The presence of the PCR product was controlled by 3% agarose gel electrophoresis. FAM-labeled single-stranded DNA was detected by means of a gel imaging system GBOX/EF2-E.

Single-stranded DNA aptamers obtained in the asymmetric reaction were separated from the PCR mixture components by using 30 kDa filter Pall Centrifugal devices (Pall, USA). Concentration of single-stranded DNA aptamers in the selected pools was determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). Isolated aptamers were used in subsequent rounds of selection and stored at −20°C.

During the second and all subsequent rounds of selection, the aptamer pool, obtained in the previous round, was initially tested for negative selection using tissue lung cancer, glioblastoma, pastopathy, healthy breast tissues adjacent to the tumor or benign breast tumor tissues. Before each round a pool of aptamers

was denatured at 95°C for 5 min, and renatured on ice for 10 min to generate aptamers of desired conformation. A suspension of target cells was incubated for 30 min with masking yeast RNA (final concentration 1 mg/mL), then with the aptamer pool from the previous round (final concentration of 200 nM). After incubation, the mixture was centrifuged for 10 min at 5000 *g* and the supernatant with unbound aptamers was collected. The supernatant was used for positive selection with breast cancer tissue samples exactly as in the case of the first round with one exception: aptamers obtained after the negative selection were used instead of a single-stranded DNA library.

Other rounds of selection were carried using the same scheme as in the second round, alternating negative and positive selections. Eleven rounds of selection to breast cancer cells were performed. The aptamer pool obtained after the sixth round of selection was used for two additional rounds of negative selection with benign breast tumors performed by the same protocol as the other negative selection procedures, but without intermediate PCR amplification. Selection of DNA aptamers by using the postoperative material resulted in generation of 12 DNA aptamer pools.

Selection of the Most Specific and High-Affinity Pool of DNA Aptamer

The most specific and high-affinity pools of DNA aptamer were selected by their ability to bind to breast cancer and benign tumor cells. The tissue samples were prepared in the same way as for selection. After washing, cells were diluted in 2 mL of phosphate buffer and passed through a filter (70 μm). Cells were counted in the Goryaev's chamber. The cell suspension containing 3×10^5 cells was incubated in 100 μL of phosphate buffer for 30 min with yeast RNA (1 mg/mL) and then the aptamer pool or a DNA library (final concentration 50 nM) were added as control. The mixture was incubated at room temperature on a shaker for 30 min. An intact cell suspension without added oligonucleotides was used as a negative control for basal cell fluorescence.

Fluorescence of aptamer pools bound to cells was determined on the cytometer Cytomics FC 500 (Beckman Coulter, USA) and the fluorescence microscope Axioscop 40 (Carl Zeiss, Germany).

RESULTS AND DISCUSSION

Typically aptamers are generated to known biomarkers (recombinant proteins) or cell lines. Several aptamers for breast cancer have been described in the literature, but their effect has been demonstrated only in model experiments, and their applicability for real clinical samples has not been investigated so far [16, 17]. The aptamer LXL-1-A is the only exception; however, its selectivity towards clinical samples was not high enough as it stained only 76% of metastatic breast cancer tissue samples and 39% of non-metastatic breast cancer tissue samples and contacted with 8% of samples of healthy breast tissues [17]. Nevertheless, since aptamer selection is based on established criteria for selecting the target receptor and because of greater availability of recombinant proteins and cell lines for researchers, the list of possible candidates for biomarkers often lacks potentially unique tumor marker proteins.

Aptamers to several protein biomarkers for breast cancer have been described in the literature; these include mucin 1 (MUC1) [18], epidermal growth factor 2 (HER2) [19], periostin [20], and the MDA-MB-231 metastatic breast cancer cell culture [17]. Aptamers obtained for cell lines and recombinant proteins were less effective in clinical samples than in model experiments [16, 17].

For generation of aptamers capable of selective recognition of tissues, circulating tumor cells, tumor markers in plasma, we used breast tissue samples obtained during surgical operations. For aptamer selection we have used the method, which has been successfully used for the selection of aptamers to human lung cancer tissues [14]. Using this method it was possible to obtain DNA aptamers that were able to recognize new lung cancer biomarkers, discriminate lung cancer circulating tumor cells from circulating cells of other types of cancer [14], stain histological tissue sections [13]. However, selection of aptamers to complex multi-component targets is always problematic, and to achieve high selectivity of aptamers during negative selection it is crucially important to use those cells, molecules, bacteria, viruses, and even plastic with which bonding must be avoided. Aptamers for breast cancer should not bind to healthy breast tissue, benign breast tumors and tissues with mastopathy, healthy breast tissue adjacent to the tumor, and tumor tissue of other localizations, so these tissues have been used in our selection procedure (Fig. 1).

Another problem related to aptamer selection includes insufficiently effective amplification of complex mixtures, which in reality aptamer pools do represent, even under optimal conditions.

In the case of aptamer selection, emulsion PCR appears to be more efficient than classical PCR [21–23]. In the complex mixture of various oligonucleotides the sequences with the simplex tertiary structure are amplified first as easier accessible to the enzyme; this results in reduced number of possible aptamers candidates due to complexity of their structure and high content of G- and C-nucleotides [23], especially in the first rounds of selection. In practice, this looks as a loss on the gel band characteristic to the aptamer pool and reducing aptamers binding to the target. In the case of emulsion PCR or correct conditions of open PCR major aptamer candidates are restored after the third round [23]. During selection of aptamers to breast cancer tissues, DNA aptamers steadily disap-

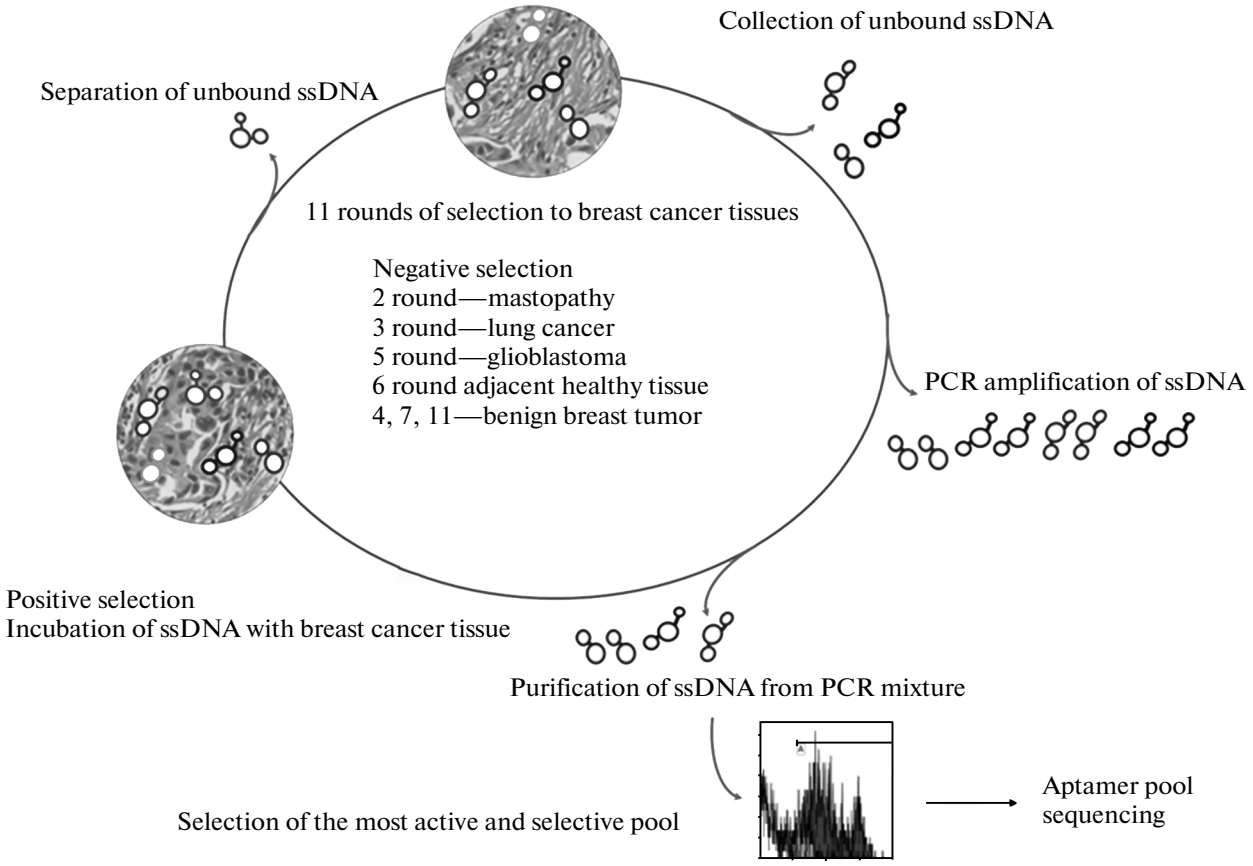

Fig. 1. Selection of DNA aptamers to breast cancer tissue (breast cancer) with negative selection to the tissue with marked mastopathy, benign breast tumors, lung cancer, brain glioblastoma, and adjacent healthy tissues.

peared (disappearance of characteristic bands on the gel) at the 3rd round of the selection and selection had to be started again. We managed to overcome these difficulties and to optimize the PCR amplification by changing PCR buffer and by adding Enhancer-1 as described in detail in the Materials and Methods section.

Among 11 pools of aptamers, the pool of the sixths round demonstrated the best binding characteristics. All the aptamer pools were tested for their ability to bind to breast cancer cells, benign tumor and healthy breast tissue. Since different types of mammary tumors are characterized by significant differences in biochemical parameters, the structural organization of tissues, protein biomarkers, it was ultimately important to select the aptamer pool with the best binding parameters towards different types of cancer tissue. DNA aptamers of all rounds of selection demonstrated good binding to the breast cancer cells of patient B (Fig. 2). It should be noted that this patient had a primary malignant tumor that did not develop from the benign tumor, as in the cases of tumors of other two patients. In patients A and C the malignant core was formed from a benign tumor. Cancer cells of patient C bound only aptamers after the sixth round of selection, while benign tumor cells of this patient demonstrated poor binding of these aptamers. In specimens of benign and malignant breast tumors from patient A, cancer cells demonstrated better aptamer binding than benign tumor cells; however, cancer cell binding of aptamers was just 23%. It is reasonable to suggest that breast cancer cells in patient A are low differentiated and less malignant.

Thus, analysis of cell binding with pools of aptamer obtained during different selection rounds and performed by flow cytometry revealed that all DNA aptamer pools demonstrated binding to benign breast tumors cells that corresponded to the level the DNA-library or even below. The only exception was the sixth pool of DNA aptamers binding, which demonstrated significant binding to the breast benign tumor cells; however, even in this case the aptamer binding was several fold lower than binding to the breast cancer cells (binding of DNA-aptamers with benign tumor was 18%, with the healthy tissue, adjacent to the tumor—16%, while binding to breast cancer cells reached 55–80%). These studies have shown possible presence of oligonucleotide sequences nonspecifically bound to breast tumor cells in the pool of DNA aptamers obtained after the sixth round of selection.

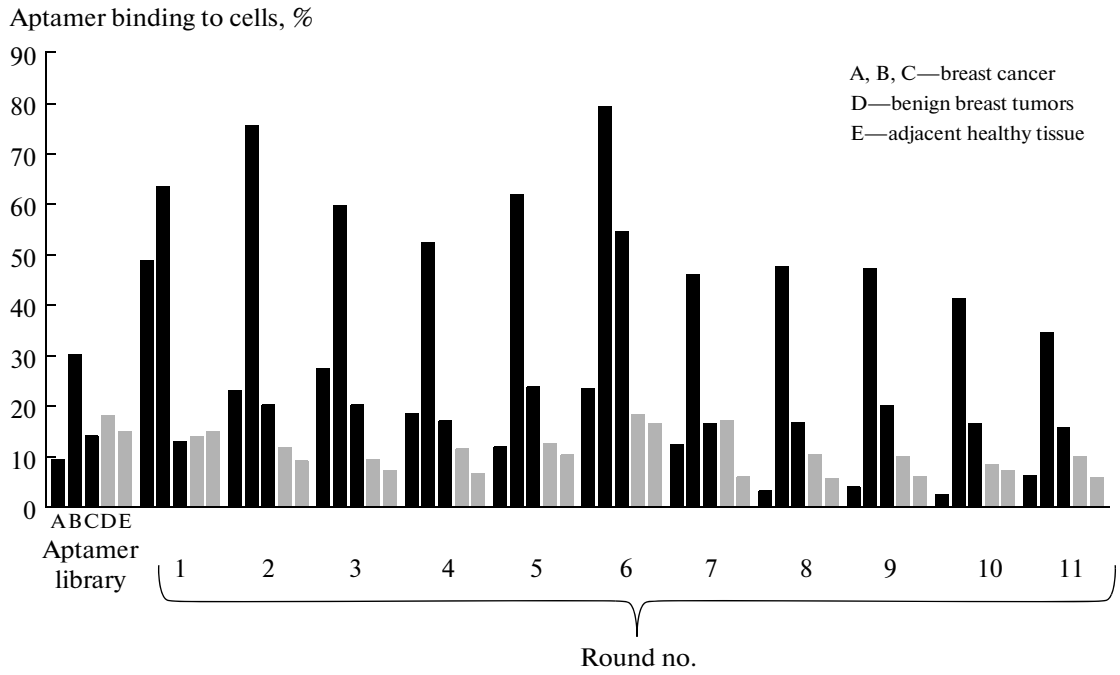

Fig. 2. Selection of the DNA aptamer pool with the best binding parameters towards breast cancer cells (breast cancer) in comparison with benign breast tumors and adjacent healthy tissue by flow cytometry. Binding of aptamers to the cells isolated from the tissues of 3 patients—A, B, C. Breast cancer (samples from patients A, B, C), benign breast tumors (D) (from patient A) and adjacent healthy tissue (E) (from patient C).

On the other hand, binding of aptamer to benign tumor cells of patients with accompanying breast cancer suggests the presence of tumor markers in surrounding non-malignant and adjacent healthy tissues, such as estrogen and/or progesterone receptors or single cancer cells.

It should be noted that the selection of aptamer pools was carried using tissue samples of 11 patients with breast cancer, 8 benign tumor tissues taken from the same patients as the malignant tissue. There were the following exceptions: round 3, in which lung cancer tissue samples were used; round 5 performed using benign tumor tissues and brain glioblastoma; round 6 performed using healthy breast tissue. This possibly explains that aptamer of this pool (6) demonstrated the best binding characteristics and selectivity.

In order to increase the selectivity of the pool of DNA aptamers obtained after sixth round of selection and demonstrated the best binding characteristics we performed two additional rounds of non-SELEX 6+ negative selection (without intermediate amplification) to a benign tumor in patients without signs of malignant transformation. Additional rounds of negative selection significantly improved selectivity of DNA aptamers obtained after 6+ round of selection. For example, binding round 6+ aptamers to benign tumor cells decreased from 23% to 12% (Fig. 3, A2), while binding of round 6 and round 6+ aptamers to glioblastoma cells did not exceed 5% (Fig. 3, A3). However, additional selection influenced aptamer binding to breast cancer cells: it decreased from 78 to 63% (Fig. 3, A1). Perhaps the decrease in the number of nonspecific sequences removed from the pool during negative selection resulted in the decreased fluorescence of the aptamer pool with breast cancer samples, but increased selectivity of this pool.

Data demonstrated aptamer binding to breast cancer and benign tumor tissues obtained using fluorescence microscopy confirmed the results of flow cytometry experiments; they showed selectivity and the DNA aptamer pool from the sixth round of selection, which further increased after the additional negative selection (Fig. 3, B1–D2).

CONCLUSIONS

Lack of sensitive and specific molecular markers of breast cancer complicates early diagnostics of this disease and thus promotes increases rates of mortality from this disease due to metastasis. Results of recent studies suggest a possibility of detection of tumor-associated proteins in biological fluids by using aptamers. Aptamers specifically bound to their molecular targets are to perform their highly sensitive identification.

We have described here a method of generation of DNA aptamers to breast tumor tissue using a tissue-SELEX technology and postoperative biological samples. Typically, aptamers are prepared using recombinant proteins or cell cultures. However, conformations

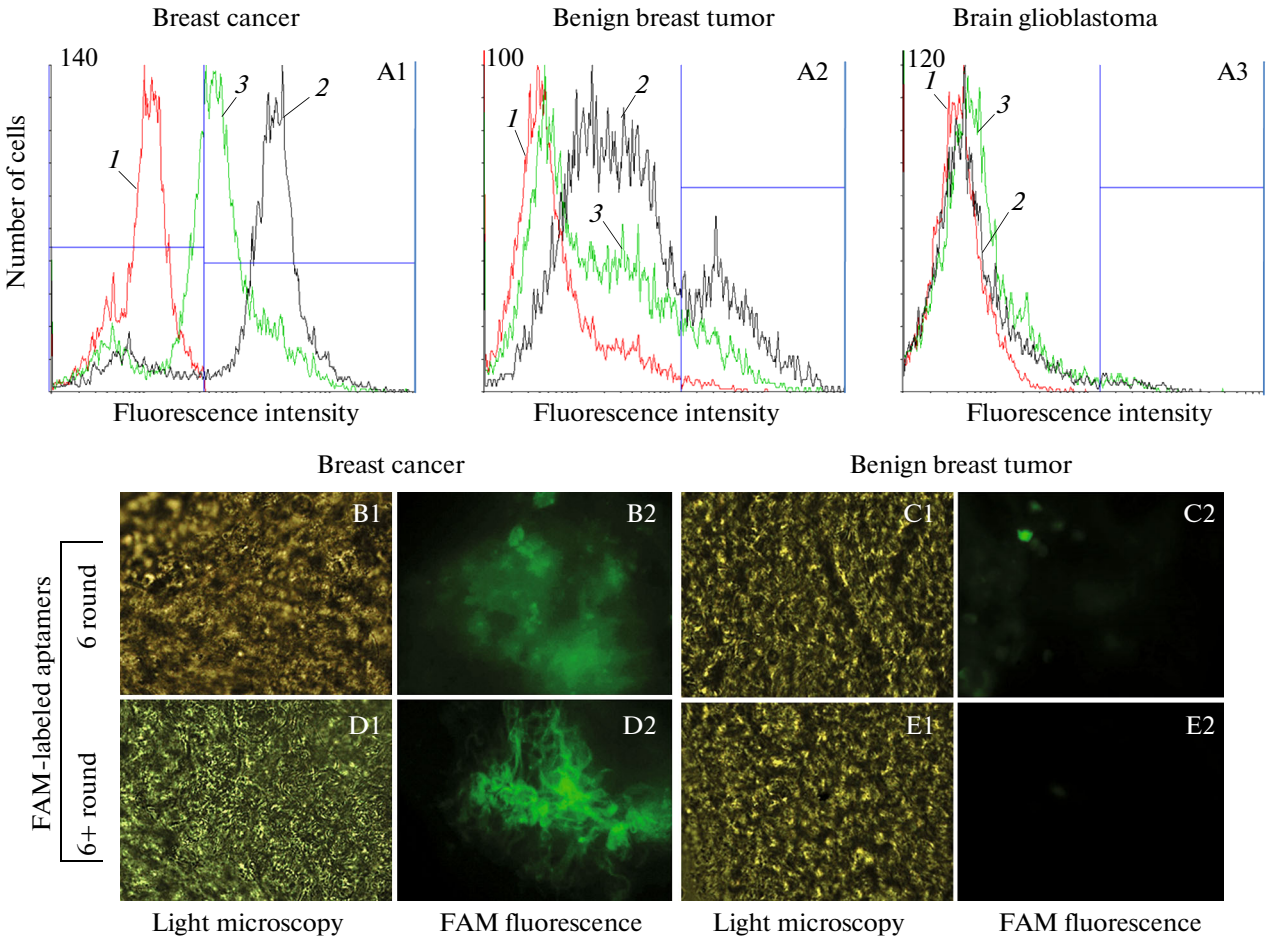

Fig. 3. Binding of aptamer obtained after the 6th round of selection before and after 2 additional rounds of negative selection 6+ to cells isolated from tissues: A1—breast cancer, A2—benign breast tumors and A3—glioblastoma, determined by flow cytometry. The histograms show (*1*) intact cells, (*2*) binding of aptamers after the 6th round of selection to cells, (*3*) binding of aptamers obtained after 6+ round of selection to cells. Binding of aptamers 6 and 6+ rounds with breast cancer tissues (B, D) and benign breast tumor (C, E) determined by fluorescence microscopy. 1—light microscopy, 2—fluorescent microscopy (fluorescent label FAM). Magnification 100×.

of intact proteins from tumor and healthy tissues may differ; this decreases specificity of resultant aptamers. In addition, phenotypes of breast cancer cell cultures differ from real samples obtained from breast cancer patients. Furthermore, tissue and tumor phenotypes may also differ in various patients. Consequently, aptamers obtained to recombinant proteins and cell cultures, may be insufficiently selective for real tumor-associated proteins in breast cancer patients. In this study using postoperative breast cancer materials derived from different cancer patients, taken as a positive target and also tissue samples of benign breast tumors, mastopathy and tumors of other types used as negative targets, we obtained a pool of DNA aptamers to breast cancer cells. The resultant DNA aptamers can be used to search for protein biomarkers of breast cancer, early diagnostics of the disease, as well as for the development of means for targeted delivery of anticancer drugs and anticancer therapy.

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